In-vivo antimalarial activities of crude extract and solvent fractions of the roots of *Clematis simensis* Fresen.(Ranunculaceae) in *Plasmodium berghei* infected mice.

A thesis Submitted to the Department of Pharmacology, School of Pharmacy in partial fulfillment of the requirement for the Master of Science degree in Pharmacology.

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This is to certify that the thesis prepared by Maru Asmare, entitled "*In-vivo* antimalarial activities of crude extract and solvent fractions of the roots of *Clematis simensis* Fresen.(Ranunculaceae) in *Plasmodium berghei* infected mice" and submitted in partial fulfilment of the requirements for the Degree of Master of Science in Pharmacology complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

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List of Abbreviations and Acronyms

ACTS- Artemisinin Based Combination Therapy

AIDS- Acquired Immuno Deficiency Syndrome

ANOVA- Analysis of Variance

DNA- Deoxyribonucleic Acid

G6PD-Glucose-6- Phosphate Dehydrogenase

HIV-Human Immuno Virus

ITN-Insecticide-Treated Nets

KAHRP - Knob-Associated Histidine-Rich Protein,

MST-Mean Survival Time

OECD- Organization for Economic Cooperation and Development

PCV- Packed Cell Volume

PfCRT- P. falciparum Chloroquine Resistance Transporter

PfEMP1- *P. falciparum* Erythrocyte Membrane Protein -1

PfEMP2- *P. falciparum* Erythrocyte Membrane Protein -2

PfEMP3 -*P. falciparum* Erythrocyte Membrane Protein -3

RDTs- Rapid Diagnostic Tests

RNA-Ribonucleic Acid

SEA-South East Asia

SEM- Standard Error of the Mean

SPSS- Statistical Package for Social Sciences

SSA- Sub-Saharan Africa

WHO- World Health Organization

Abstract

Background: Medicinal plants have contributed significantly to current malaria treatment. Alarming signs of emerging resistance to currently available drugs has necessitated the search for new plant based antimalarial agents and several plant based, pharmacologically active anti-malarial compounds have been isolated. This study was conducted to validate the traditional usage of *C.simensis* for treating malaria in the traditional health care system of Ethiopia.

Methods: The roots of *C.simensis* were collected around Gondar town, 738km North West Ethiopia. After collection, the plant materials were identified by a taxonomist, dried under shade and crushed to powder. The powdered roots were extracted by maceration using 80 % methanol and fractionated using n-hexane, chloroform and distilled water. Acute toxicity study of the crude extract was carried out in female Swiss albino mice. The in vivo anti-malarial activities of crude, aqueous, chloroform and n-hexane fractions (100, 200,400 and 600 mg/kg) of *C.simensis* roots against a chloroquine (CQ) sensitive strain of *P. berghei* strain ANKA was evaluated using the four-day suppressive and curative test procedures. Parameters such as parasitemia, packed cell volume, rectal temperature, body weight and survival time were then determined.

Results: The 4-day suppressive oral administration test of the 600mg/kg methanolic crude extract ($P < 0.001$) and 400mg/kg chloroform fraction ($P < 0.01$) showed significant parasitemia reduction compared with the negative control. And their respective percentage suppressions were 53.54 and 26.45. Additionally, the curative test of chloroform fraction achieved significant parasitemia reduction for 600mg/kg ($P < 0.001$) and 400mg/kg ($P < 0.05$) in relation with negative control. The activities of all the extracts on *P. berghei*-infected mice were increased in a dose-dependent manner. Its oral LD50 was found to be greater than 2000 mg/kg.

Conclusion: The result revealed the methanolic crude extract and chloroform fraction of *C.simensis* roots have anti-malarial activities against *P. berghei* in an animal model and supports the use of the plant to combat malaria in Ethiopian folk medicine. Further work is necessary to isolate, identify and characterize the active principles from the plant material.

Keywords: *In-vivo*, Anti-malarial, *C.simensis*, crude extract, solvent fractions *P. berghei*, *4*-day suppressive, curative and phytochemicals.

1. Introduction

Malaria is a vector borne infectious disease caused by hemo- protozoan of the genus Plasmodium, phylum *Apicomplexa* and family *Plasmodidae(*1, 2). The disease is transmitted by the bite of infected adult female Anopheles mosquitoes (3). It is a critical health problem in the world and one of the top three killer infectious diseases (HIV/AIDS, TB and malaria,) globally(4).

1.1. Epidemiology and economic impact of malaria

Knowing the features of malaria in endemic populations is a key part in quantifying and monitoring the impact of current and past control efforts (5). Malaria was endemic in 104 tropical and subtropical countries, including parts of the Americas, South-East Asia and Africa (5-7). and has a major impact on global public health and the economy, with an estimated 3.3 billion people at risk in 2011(WHO)(2, 8).

According to WHO-2012, the disease is preventable and treatable, yet annually there were 207 million estimated cases from which 627,000 deaths of malaria and majority of cases (80%) and deaths (90%) accounting in Sub-Saharan Africa-SSA (9-11). In 2013, WHO estimated that malaria caused about 198 million illnesses and 584 000 deaths globally. Africa is the most affected continent with 90 % of all malaria deaths mainly in children under 5 years old (8). There were 214 million new cases of malaria in 2015 globally (WHO)-Africa accounted - 88%, South - East Asia-10% and Eastern Mediterranean (2%). From 438,000 malaria deaths worldwide under-fives accounting 306, 000(2, 12).Malaria has placed a heavy economic burden on health systems especially in Africa, on average it costs 300 million US Dollars for case management per year(12).

Malaria is one of the leading health problems in developing countries including Ethiopia. In Ethiopia malaria is unstable and seasonal as the country's heterogeneous topography and climate conditions(13). Malaria affects more than 68% of the population in Ethiopia, a country of 94 million people in 2013. Across the nine administrative regions, areas that lie below 2000 m altitude are malarious. The high malaria transmission season overlaps with the cultivation months; hence it has a deleterious effect on agricultural production (14, 15) Studies showed,

history of travel is a risk factor for malaria in Ethiopia. The major driving force for macro-level population movements is seasonal agricultural labor (10).

1.2. Life cycle and pathophysiology of malaria

1.2.1. Life cycle of malaria

Infection is initiated when a parasitized Anopheles mosquito injects tens to hundreds of sporozoites into the dermis of the host (16).Sporozoite stage traverses multiple host skin cell types, taken up by the blood stream and transported to the sinusoids of the liver and each parasite establishes itself in a single hepatocyte and infect it (17). In most *Plasmodium* species, all parasites then undergo many rounds of replication for 5-12 days, depending on spp., in a single hepatocyte as liver schizonts and ultimately form tens of thousands of infectious merozoites (17, 18).

P. vivax and *P. ovale* have a dormant stage, named hypnozoite that can remain in the liver for several months to years before the development of pre-erythrocytic schizogony resulting in relapses of malaria infection. *P. malariae* can result in long-lasting infections and if untreated can persist asymptomatically in the human host for many years, even a lifetime (19).Merozoites are released in to the circulation and begin the asexual blood stage where cyclic infection, replication and lytic release from red blood cells (RBCs) occur. This rapidly multiplies the parasite in to billions and causes all malaria-associated morbidity and mortality (19).

A small percentage of asexual stages terminally differentiate into sexual erythrocytic stages (3, 20).After these sexual stages are taken up by a female anopheline mosquito while ingesting a blood meal, they mate and infect the mosquito's mid gut. Further differentiation and expansion of parasites generates thousands of progeny sporozoites and that travel to the mosquito's salivary glands (21).

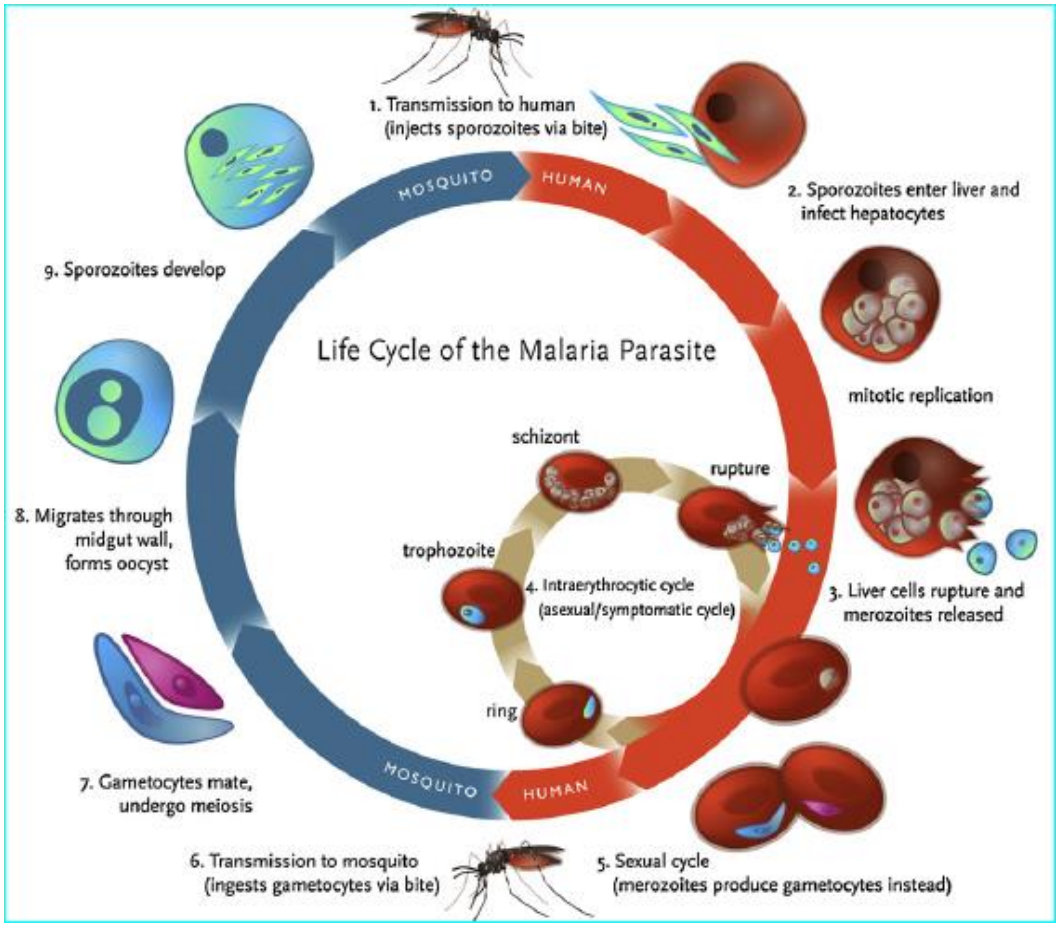

Figure 1 Life cycle of the malaria (22).

1.2.2. Pathophysiology of malaria

A lot of *Plasmodium* species infect humans and other animals, including rodents' birds and reptiles (23). Of the five Plasmodium SPP.(*P. falciparum*, *P. vivax*, *P .ovale* and *P. malariae*) that infect humans, *P. vivax* and *P. falciparum* cause the majority of cases, and *P. falciparum* is the most virulent and responsible for most of the deaths (24). (*P. knowlesi*, the fifth occasional human parasite is essentially a non-human primate malaria species found in Southeast Asia (11).

In Ethiopia, *P. falciparum* and *P. vivax* are the main species accounting about 60% and 40% of malaria cases, respectively (25). More than 400 species of the *Anopheles* mosquito have been described and about 70 of these species are potential vectors of malaria that affect humans (23).The dominant malaria vector in Ethiopia is *Anopheles arabiensis*; and *Anopheles pharoensis*, *Anopheles funestus* and *Anopheles nili* are secondary (25).

The pathogenesis of human malaria emanates from various host and parasite factors that simultaneously influence the severity and outcome of the disease. Important pathophysiologic features include the induction of pro-inflammatory cytokines, the sequestration of *P. falciparum*-infected erythrocytes in the microvasculature, and anemia due to suppression of erythropoiesis. The destruction of infected and uninfected erythrocytes further compromises oxygen delivery and exacerbates disease pathogenesis (26).

In *P. falciparum* the blood stage schizonts disappear from the circulation because they adhere to host ligands on endothelial cells of the microvasculature of various organs throughout the body including the brain and placenta, through parasite proteins expressed on the infected erythrocyte surface leading to sever pathogenesis such as cerebral malaria or adverse effects during pregnancy(placental complications)(27). Important virulence properties of *P. falciparum* are sequestration and the expression of parasite-derived antigens (comprised of novel parasite-derived proteins including *P. falciparum* erythrocyte membrane protein 1 (PfEMP1)) on the surface of infected erythrocytes and the adhesion of it to the vascular endothelium or to uninfected erythrocytes to form rosettes which results in excessive microvasculature obstruction. (The clustering of mature infected erythrocytes to uninfected erythrocytes, known as rosetting (26).

The proteins of the *P. falciparum stevor* and *rifin* multi-gene families are involved in infected erythrocyte interactions such as rosetting and tissue sequestration. *Stevor*-induced rosetting provides a growth advantage by protecting merozoites from invasion by blocking antibodies(28).

A feature of infected erythrocyte that enables *P. falciparum* to sequester is the expression of knob structures on the infected erythrocyte's membrane. The structural component of knobs is the knob-associated histidine-rich protein, KAHRP (major), PfEMP2 and PfEMP3. KAHRP enables reduced membrane deformability of both infected and uninfected erythrocytes compromising their passage via partially obstructed capillaries and venules and shortens RBC survival(26).

Large proportions of these exported proteins encoded by multi-copy gene families involve in antigenic variation and immune evasion. These Plasmodium proteins are involved in uptake of nutrients, formation of membranous structures, and protein trafficking and mediating adherence

of infected erythrocytes to host cell receptors. These proteins operate as ligands that bind to host cell receptors on the capillary endothelium resulting in infected erythrocytes tissue sequestration, which is believed to prevent infected erythrocytes from being removed by the spleen (28).

The parasite proliferation within the host's erythrocyte takes place using hemoglobin as nutrition source. Massive degradation of the hemoglobin within the digestive vacuole of the parasite generates large amount of toxic soluble heme. To protect itself, the parasite polymerizes the heme to an insoluble crystalline pigment known as hemozoin, which is sequestered in the food vacuole. Hence, hemozoin synthesis is an indispensable process and the target of action of several antimalarials (29)

1.3. Diagnosis and Management of malaria

1.3.1. Diagnosis of malaria

Early and effective malaria diagnosis is the main thrust for control and management of the disease. Till now peripheral blood smear remains the gold standard for the diagnosis of Plasmodium species and to determine the disease burden(29). In many hospitals and health centers diagnosis is carried out by microscopy, whereas the health posts employ rapid diagnostic tests (RDT), relatively require no skilled personnel, for P. falciparum and P. vivax diagnosis(25). The tests contain antibodies conjugated to latex particles that bind to the circulating antigens(29).

Some malaria parasites lack the histidine rich protein 2 (HRP2) proteins, the most common target antigen used in RDTs for detection of *P. falciparum*. Hence, the parasites can escape detection by rapid diagnostic tests and subsequent treatment with an ACT. This prevents a patient from receiving appropriate treatment, enables the parasite to survive, reproduce and increase in relative frequency (30). RDTs do not allow quantification and differentiation of different *Plasmodium* species other than *P. falciparum* and *P. vivax (25).*

Recently molecular detection of Plasmodium with higher sensitivity is gaining paramount importance for accurate detection. Polymerase chain reaction (PCR) detects parasite DNA and can identify infections below the threshold of detection for microscopy and RDTs in blood. The PCR approach identifies the species-specific Plasmodium DNA by amplifying the 18s rRNA region of the parasite (highly sensitive but too complex)(29). Mixed infections are best detected

by PCR amplification techniques; they may be underestimated with routine microscopy(18). Currently, PCR is mainly used in drug efficacy studies. Neither is used for clinical managements (31).

1.3.2. Management of malaria

Malaria case management including early diagnosis and prompt effective treatment, remains a vital component of malaria control and elimination strategies (18).The main guiding factors those need to be focused during treatment are; the type of infecting *Plasmodium* species, clinical status of the patient and drug susceptibility of the infecting parasites as determined by the geographic area where the infection was acquired and the previous use of antimalarial medicines (32).

1.3.2.1. Treatment of uncomplicated *P. falciparum* malaria

Most of the countries with *P. falciparum* malaria have been adopted ACTs as a first-line treatment. According to WHO recommendation, uncomplicated *P. falciparum* malaria should be treated with an ACT (both children and adults) (18). In areas where there is artemisinin resistance and in areas targeted for *P. falciparum* elimination, a single primaquine dose (0.25 mg/kg) is given for all patients with confirmed *P. falciparum* on the first day of their ACT treatment. A single dose of primaquine was being utilized for gametocidal treatment of *P. falciparum* cases in 39 low-transmission countries for the reduction of malaria transmission (8, 18)

Even though use of primaquine in radical curative regimens needs glucose-6- phosphate dehydrogenase (G6PD) testing, a single low dose of primaquine as a *P. falciparum* gametocytocide has proved safe and well tolerated in G6PD deficient patients (2).

In the previous years, chloroquine was effective in treating almost all cases of malaria. In 2013, ACTs had been adopted as national policy for first-line treatment in 79 out of 87 countries where *P. falciparum* is endemic; chloroquine is used in 10 Central American and Caribbean countries where it remains efficacious (8, 18).

1.3.2.2. Treatment of severe malaria

Mortality rate from untreated severe malaria (particularly cerebral malaria) is about 100%. But if prompt, effective antimalarial treatment and supportive care is employed, the overall rate falls to 10 - 20%.There are two classes of drugs for parenteral treatment of severe malaria: artemisinin derivatives (artemether or artesunate) and cinchona alkaloids (quinidine and quinine). Parenteral artesunate is the treatment of choice for all severe malaria as it is simpler and safer to use (18).

Adults and children with severe malaria (including infants, pregnant women in all trimesters and lactating women)must be treated with intravenous or intramuscular artesunate for at least 24 h and until they are able to take oral medications. Once a patient has received at least 24 h of parenteral therapy and can tolerate oral therapy, complete treatment with 3 days of an ACT. If parenteral artesunate is not available, we can use artemether in preference to quinine for treating children and adults with severe malaria (18).

1.3.2.3. Treatment of uncomplicated non-*falciparum* malaria

Treatments for unknown blood stage malaria species infection is the same as for uncomplicated *P. falciparum* malaria. In chloroquine effective areas, four of the plasmodium spp. malaria are treated with either ACT (except pregnant women in their first trimester) or chloroquine in both adult and children (18). In areas where resistance to chloroquine has been detected, four of the plasmodium spp. malaria is treated with an appropriate ACT (8). Pregnant women in their first trimester who have chloroquine-resistant *P. vivax* malaria is treated with quinine (18).

For the avoidance of relapses in *P. vivax* or *P. ovale* chloroquine and ACT must be combined with a 14-day course of primaquine (except pregnant women and infants aged less than 6 months) by taking a risk of hemolysis in to consideration in patients with glucose-6- phosphate dehydrogenase (G6PD) deficient(29).In 2013, 55 out of 56 countries with transmission of *P. vivax* malaria, primaquine are being utilized for the treatment of the hypnozoite stage of *P. vivax* malaria (8).

1.3.2.4. Treatment of uncomplicated *P. falciparum* malaria in special risk groups

Pregnant women with uncomplicated *P. falciparum* malaria during the first trimester should be treated with 7 days of quinine + clindamycin. Infants weighing < 5 kg with uncomplicated *P. falciparum* malaria should be treated with ACT at the same mg/kg body weight target dose as for children weighing 5 kg. In people who have HIV/AIDS and with uncomplicated *P. falciparum* malaria, we must avoid artesunate + SP if they are being treated with co-trimoxazole, and artesunate + amodiaquine if they are being treated with efavirenz or zidovudine (18).

1.3.2.5. The role of Traditional medicine in malaria treatment

People have been used medicinal plants from time immemorial (11). There are 1200 plant species from 160 families used to treat malaria globally (33, 34).They are safe, economical, less toxic and a reliable key natural source of drugs all over the world. Phytochemicals are naturally occurring in the medicinal plants, leaves, roots and vegetables that have defense mechanism and protect us from various diseases. They are primary (chlorophyll, proteins and common sugars) and secondary (terpenoids, alkaloids and phenolic etc.) compounds (35). Seasonal variations can affect the chemical constituent of the plants and thus their biological activity, maximum at the time of flowering and then declines at the beginning of the fruiting stage (36).

Populations in most endemic countries are highly dependent on the use of plant-based complementary medicine for therapy, as most of them lack the access to modern healthcare facilities. According to the World Health Organization (WHO) estimates, arround 80% of the populations in developing countries including Ethiopia use traditional medicines (37, 38). Drugs derived from natural sources, especially plants, represent majority of the pharmaceutical market, particularly for malaria therapy. Their advantage for the development of new drugs arises from the synergistic interactions of their components and their innate affinity for biological receptors. Justification of these principles could provide pharmacological basis for further development of plant-based traditional medicine as a reliable therapeutic tool(39).

In Ethiopia, the use of traditional medicinal plants is widely practiced. Traditional therapies are the most important and sometimes the only source of remedies for most of the Ethiopian population and 95% of which are plant origin(40).

1.3.2.6. Prevention, Chemoprophylaxis and Control of malaria

Malaria control becomes an increasingly important international focus(29). Malaria cases can be prevented by vector control (stopping mosquitoes from biting human beings), by chemoprevention (providing drugs that suppress infections) or, potentially, by vaccination (30).

Studies in malaria endemic areas of SSA- countries, including Ethiopia, showed that the combination of widespread distribution of long-lasting insecticidal nets to all households (reduce malaria incidence rates by 50%, and mortality rates by 55% in children aged under 5 years in SSA) indoor residual spraying (IRS), and nationwide distribution of artemisinin-based combination therapy (ACT) in the public sector were major control tools associated with substantial declines of malaria (29, 30).

Chemoprevention is vital in pregnant women and young children(29).In SSA, intermittent preventive treatment of malaria in pregnancy by sulfadoxine-pyrimethamine (SP) has been shown to reduce low birth weight, perinatal mortality and maternal anemia.

Intermittent preventive treatment in infants with SP provides protection against clinical malaria and anemia; but, as of 2015, no countries have been implemented this policy.

Seasonal malaria chemoprevention with amodiaquine, AQ + SP for children 3–59 months old decreases the incidence of clinical attacks and severe malaria by about 80% and could avert millions of cases and thousands of (30).

1.4. Vector insecticide and antimalarial drug resistance

Resistance of malaria vectors to the four insecticide classes currently being used in insecticide-treated nets (ITNs) and IRS threatens malaria prevention efforts. from the 73 malaria endemic countries that provided monitoring data to WHO for 2010 onwards, 60 of them reported resistance to at least one insecticide in one malaria vector from one collection site, and 50 reported resistance to ≥ 2 insecticide classes. Resistance to pyrethroids - the only class currently used in ITNs-is the most commonly reported (30) .

Resistance to all antimalarial drug classes seems to originate from a specific area of the world South East Asia (SEA)-mainly human migration especially young who travel extensively in forested areas and forest-dwelling mosquitoes render conventional vector control methods less effective, resulting in a greater dependence on drugs for malaria control(2).

Recent genetic studies analyzing microsatellite markers surrounding the *P. falciparum* chloroquine resistance transporter gene (*pfcrt*) (2), 424-peptide transmembrane protein involved in nutrient transport and osmotic balance across the parasite digestive vacuole membrane(41).,have confirmed that chloroquine resistance did spread from Asia to Africa. Using the same technique the main form of Sulfadoxine pyrimethamine resistance (triple 51, 59 and 108 dihydrofolate reductase (*dhfr*) gene mutations) was shown and also spread from SEA to Africa (2).

The resistance of *P. falciparum* to artemisinin has been detected in five countries of the Greater Mekong sub region, first detected in 2008 (30). The use of oral artemisinin based monotherapy medicines threatens the long-term usefulness of ACTs, because it fosters the spread of resistance to artemisinin. WHO recommends that oral artemisinin based monotherapies is withdrawn from the market and marketing of the products should be stopped (8).

Previously, many countries have been conducting therapeutic efficacy studies for antimalarial medicines. Such studies remain the gold standard for guiding antimalarial treatment policies. But, recently, a molecular marker of artemisinin resistance was identified for specific point mutations in the *Kelch13* (chromosome 13, K13) i.e., propeller domain of *P.falciparum* protein found to be associated with delayed parasite clearance (> 5 h). This may open new possibilities for tracking resistance to artemisinin (2, 8).

1.5. Development of malaria vaccine

Malaria vaccine research began in the 1930s by focusing on inactivated or killed parasites that failed to generate a protective immune response but still antimalarial immunity is poorly understood(42). Malaria is caused by more complex parasites in terms of their biology than the viruses and bacteria for which we have vaccines. They pass through multiple stages of development in the human host expressing hundreds of unique antigens. This complexity makes

it more difficult to develop a vaccine for parasites than for viruses and bacteria, since an immune response targeting one stage may not offer protection against diverse later stage antigens. In addition, depending on the life cycle stage and whether the parasite is extra-or intra-cellular, antibody and/or cellular immune responses provide protection(43). To date, malaria vaccination strategies have mainly focused on sporozoites and liver stages or blood stage of infection targeting stage specific parasite antigens(16). But, success has been limited with these stage-specific approaches, raising the question to give greater emphasis on multi-stage vaccination approaches(16).

In July 2015, the most advanced malaria vaccine candidate and the first to undergo large-scale phase 3 evaluations in Africa, RTS, S/AS01 made by GlaxoSmithKline received a positive opinion from European regulators for the first time(44) .This targets the pre-erythrocytic stage of P.falciparum inducing humoral and cellular immune responses to the circumsporozoite protein (CSP) present on the surface of sporozoites and liver stage schizonts. The development of this vaccine is initiated in the late 1980s, and invested about $610 million to date(44, 45).

RTS, S reduced clinical incidence by 39% and severe malaria by 31.5% among children aged 5–17 months who completed four doses. WHO recommended that RTS, S be implemented on a pilot scale in parts of 3-5 SSA countries in 3–5 years time. The first phase of vaccination is expected to commence in 2018. RTS, S is being considered as a complementary malaria control tool in Africa that could potentially be added to, rather than replace, the core package of proven malaria preventive, diagnostic and treatment interventions (30).

1.6. Investigational antimalarial agents

The lethal impact of *pfent1* knockout for parasites which are grown in purine concentrations found in human blood suggests that it might be a target for the development of novel antimalarial drugs, but, no inhibitors of *pfent*1 have been identified to date (46).

The new drugs in development are those with known chemical structures and/or mechanism of action with enhanced properties- tafenoquine (an 8-aminoquinoline), artefenomel (OZ439)(a synthetic peroxide), and P218 (an antifol); and those with new chemical classes such as KAE609 (a spiroindolone) and KAF156 (an imidazolopiperazine). The threat of potentially untreatable

falciparum malaria is real, and will require radical action if malaria is to be eliminated before the available drugs become ineffective(2).

1.7. Description of the Genus *Clematis* (Ranunculaceae)

Members of the genus *Clematis* are mostly perennial woody vines, few perennial sub-shrubs, shrubs or herbs distributed worldwide, predominantly in tropical and subtropical regions. Within the subfamily Ranunculoideae of Ranunculaceae, it belongs to the tribe Anemoneae, sub-tribe Clematidinae. Family Ranunculaceae comprises 59 genera and about 1900 species. Genus *Clematis* has about 355 spp. in the world (2005)(47, 48).

There are reports on the chemotaxonomy of genes *Clematis*, using flavonoids, triterpenoid saponins, ribose, etc profiles as chemical markers for the taxonomy of the Ranunculaceae. Many species of *Clematis* from China are of medicinal significance. Various *Clematis* species have been shown experimentally to exhibit significant anti inflammatory (47, 49) and analgesic, antifungal, antitumor, cytotoxic and cyclooxygenase-2 inhibitory activities, which have been attributed to the saponins found in the genus (47).Chemical and pharmacological studies have shown that triterpenoid saponins are the main components in the genus *clematis* having a wide spectrum of biological effects (50)

The volatile constituents of the three *Clematis* species viz. *C. hirsute, C. Simensis* and *C. wightiana* showed a pronounced anti-inflammatory activities and volatiles of *C. hirsute* and *C. simensis* showed similar activities against gram-positive, gram-negative bacteria and fungi (51).The ethanol extracts of three *Clematis* species, *C. Pickeringii* (with the highest inhibitory activities), *C.glycinoides* and *C. microphylla*, inhibited the activities of cyclooxygenase-1 (COX-1), cyclooxygenase-2 (COX-2) and 5-lipoxygense (5-LOX) (52).

70% EtOH crude extract of young shoots of *C. Vitalba* was shown broad activity against pathogenic yeast and yeast-like microorganisms. After fractionating with petroleum ether, ethyl acetate and methanol, antimycotic activity has been observed only in methanol fractions and triterpenoid saponins were also isolated in this plant (53).The roots and rhizomes of genus Clematis are commonly used as an analgesic, abirritative, anti-inflammatory, antibacterial, antiphlogistic, anticancer and diuretic agent (54).

A study showed the hepatoprotective effect of *C. chinensis* extract on CCL4-induced hepatotoxicity in rats and the results of aminetransferase, aspartate aminetransferase and alanine aminetransferase have shown a significant hepatoprotective effect after treatment with *C. Chinensis (52)*. Medicinally effective chemical compounds are apparently absent in young sprouts therefore young sprouts of various *Clematis spp*. are eaten cooked as vegetable (55).

The Izoceno-Guarani of Bolivia burned the aerial parts of *C. denticulata* Vell. cabelo del angelo. over fire and inhaled the smoke to treat malaria (56).Various African herbs (e.g. *C. brachiata, C. hirsuta* and *C. oweniae*) were used to induce sneezing to clear head colds or headaches. Patients suffering from malaria and colds boiled *C.oweniae* in hot water and taken it as a tea (57).

Twenty six species of the genus Clematis have been traditionally used in various systems of medicine for the treatment of ailments such as nervous disorders, syphilis, malaria, dysentery, rheumatism, asthma, and as analgesic, anti-inflammatory, diuretic, antitumor, antibacterial and anticancer (58)

According to "*Chinese Materia Medica*" *C. terniflora DC.* Var. *mandshurica* (Rupr) Ohwi, C. *mandshurica* Rupr, *C. meyeniana* Walp, *C. uncinata* Champ. Ex Benth, *and C. uncinata* Champ. Ex Benth var. *biternata* W. T. Wang are traditionally used for the treatment of malaria *(52)*. Methanolic extract of *C.campestris* A.St.-Hil. flower, showed good *in vitro* antiplasmodial activity against *P. falciparum (59)*

1.8. Experimental plant, *C. simensis* Fresen. (Ranunculaceae)

C. simensis is one of the plants that belong to the family Ranunculaceae, which has synonyms – *C.orientalis*,L, L, and *C.altissima*, Hutc (60, 61)., "Azoareg" in Amharic(62). It is a tall climbing shrubby plant, up to ≥ 20 m. The plant grows in an altitude range of 1000-3360m (60, 61).

Figure 2- A photographs of *C. simensis*.

Ethno pharmacologically -crushed shoots of maceration used for cough treatment (63), crushed fresh leaves for tonsillitis, cellulites and leech infection treatment(for cattle) (64), leaves for wound treatment (65). Stem, tooth brush *as* antimicrobial (for *Streptococcus pyogenes* (66),leaves chewed and juice swallowed as cure for headache and colds(67, 68), squeezed leaves for hemorrhoids/tumor (69), grinding and giving a finger tip of this nasally for malaria treatment (68, 70), root/twig-homogenization orally for malaria , cough and liver disease(71). Root decoction drunk for treatment of malaria and as purgative (68). According to the people of Burundi, smoke from burning leaves is reported to have analgesic properties (56).

In addition, *in vitro*, antibacterial activities of *C. simensis* leaf extract on Enset Bacterial Wilt (*Xanthomonas campestris PV Musacearum*) in combination with other plants have showed moderate inhibitory activities against bacterial isolate in comparison with standard antibiotic-Penicillin (72). Its leaf extract also experimentally showed *in vivo* anti inflammatory and anti-nociceptive activities (49).

Apart from the above antimalarial claims of the literature no experimental studies have been reported on *in vivo* antiplasmodial effects of extracts of *C. simensis* Fresen.(Ranunculaceae)

1.9. Significance of the study

Even though malaria is among the preventable and treatable infectious diseases, it is still one of the major causes of mortality and morbidity in tropical and subtropical countries and remains a major global health concern with 207 million estimated cases and 627,000 deaths of malaria (WHO-2012) (9, 25). Antimalarial drugs currently remain the most effective tool for malaria treatment together with vector control strategies and for malaria prophylaxis. Unfortunately, the rapid spread of drug-resistant *P. falciparum* parasites is compromising the efficacy of available antimalarial drugs (73).

Containing resistance is becoming increasingly difficult as *P. falciparum* develops resistance to all antimalarial drug classes. Moreover, increasing numbers of international travelers and improved transportation facilitate the spread of resistant parasites (2). Alarming signs of emerging resistance to artemisinin derivatives threaten the now widely-used artemisinin core combination therapies (ACTs) (39), and the discoveries of new antimalarial agents are urgently needed, with broad therapeutic potential and novel modes of action, to widen the scope of treatment and to overcome emerging drug resistance in malaria parasites. Drugs that target different, preferably multiple, parasite life cycle stages are a high priority(73).

Quinine (from *Cinchona spp*.), Atovaquone (from *Tabebuia impetiginosa*) and recently introduced artemisinin (from *Artemisia annua*) and its derivatives as antimalarials demonstrate that plant species are an important source to search for new antimalarial agents (39). In addition, many plant species remain to be used in traditional medicines for the treatment of malaria and many people depend on such remedies as they cannot afford and even do not have an access to effective antimalarial drugs.

One of the approaches in the search for new antimalarial drugs is the use of more predictive traditional herbal remedies which have been served as a source of the majority of conventional antimalarial drugs. The interest in this plant is justified by its traditional claims and potential medicinal value against malaria and reported pharmacological activities of the leaf extracts for anti-inflammatory activity might have antimalarial activities (39, 68, 70, 71). The present study, therefore, focuses on investigation of antimalarial activities of crude extract and solvent fractions

of *C.simensis* root in mice infected with *P.berghei*, hoping that this will provide a base line data for further investigations on antimalarial effect of the plant.

2. Objectives of the Study

2.1. General objective

- To investigate antimalarial activities of crude extract and solvent fractions of *C. simensis* roots extract in *P. berghei* infected mice.

2.2. Specific objectives

- To undertake preliminary phytochemical screening of the crude extract of *C. simensis* roots.
- To determine the oral acute toxicity of *C. simensis* roots extract.
- To evaluate the antimalarial activities of the crude extract of *C. simensis* roots using 4 days suppressive test.
- To evaluate the antimalarial activities of the hexane, chloroform and aqueous fractions of *C. simensis* roots using 4 days suppressive test.
- To evaluate the antimalarial activities of the most active solvent fraction of *C. simensis* roots using curative tests.

3. Materials and Methods

3.1. Materials

Chemicals, drugs and reagents that were used - Absolute Methanol(Loba Chemie,India), Giemsa stain, Chloroquine Phosphate(APF, Ethiopia), benzene(Blulux laboratories (P) LTD, India), Wagner's reagent, Mayer's reagent, concentrated H_2SO_4, Glacial Acetic Acid, sodium citrate, Ethanolic Ferric Chloride(Fisher, scientific CO, USA), dilute Ammonia, Acetic Anhydride, aqueous hydrochloric acid, copper acetate solution, Chloroform-99.8%(Atico, India).

3.2. Plant material collection and identification

The fresh roots of *Clematis simensis* Fresen were collected around Gondar town, 738km North West Ethiopia, in December, 2016 and the plant specimen was identified and authenticated by Mr. Abiyu Enyew, Department of Biology, college of Natural and Computational Sciences, University of Gondar and the Isotype of the specimen was deposited at their herbarium with a Voucher number of MA001 for future reference**.**

3.3. Experimental animals and parasite

Female Swiss albino mice of (age 8-9 weeks and weight of 23-28 g) breed and maintained at the University of Gondar, Department of Pharmacology and from the Ethiopian Health and Nutrition Research Institute (EHNRI) Addis Ababa, Ethiopia, were used. They were maintained under standard condition (temperature of 22 ± 3°C, relative humidity of 50-60% and 12 h light/12 h dark cycle), with pellets and water *ad libitum* in the animal house of University of Gondar, School of Pharmacy, Department of Pharmacology . Animals were acclimatized for one week to the experimental environment. The care and handling were according to the National Research Council Guide for the Care and Use of Laboratory Animals (74-76).

Chloroquine sensitive strains of *P.berghei* (ANKA) obtained from EHNRI was used. The parasites were maintained by serial passage of blood from infected mice to non-infected ones on weekly basis(77).

3.4. Extraction procedure for crude extract

The collected plant roots were cleaned with tap water and air dried at room temperature under shade after slicing it into small pieces to facilitate homogenous drying. A total of 1.9Kg of dried roots was extracted by maceration (100g of dried roots in 526ml of 80% methanol) for 72 hrs. The extraction process was facilitated by using an orbital shaker at 120 rpm. The mixture was first filtered using sieve and then withWhatman filter paper (No.3, 15cm size with retention down to 0.1μm in liquids). The residue was re-macerated for another 72 hrs twice and filtered. The combined filtrates were dried by rotary evaporator (model−RE200, UK) under reduced pressure at a temperature of 40°C (to remove methanol). The remaining aqueous part was further dried using a lyophilizer (Ningbo Scientz Biotechnology, China). After drying, a total yield of dry extract was determined and kept at −20°C until use. Portion of the crude yield (10g) was used for confirmation of the antimalarial activities and the remaining(151g) was used for fractionation(78).

3.5. Fractionation

The successive solvent-solvent extraction procedure was followed for the fractionation of the plant sample. The crude 80% methanolic extract of C.simensis 151g was suspended in 450 ml of distilled water in separatory funnels and this was further diluted with 450 ml of n-hexane. After gently shaking for 5minutes, the extract was allowed to settle for 25 minutes to be separated into two distinct layers according to their density. The upper n-hexane layer was collected and the same procedure was repeated three times until colorless n-hexane layer was obtained. After the collection of n-hexane fraction of the extract, it was fractionated with 450ml chloroform thrice the same way like that of n-hexane and the bottom chloroform layer was collected leaving the aqueous fraction at the top. The n-hexane and chloroform fractions were dried in an oven of 40^{o}C, and the aqueous fraction was further dried using a lyophilizer.(Ningbo Scientz Biotechnology, China). Furthermore, the percentage yield for n-hexane, chloroform and aqueous fractions of the plant was determined. These dried fractions were then transferred into separate vials and stored at −20°C until use (78-80).

3.6. Phytochemical screening

The secondary metabolites in the plant sample are the main concern of a research work. A preliminary phytochemical study was performed on crude extract of the roots by coloring and precipitation assays using standard procedures to relate the antimalarial activity of the plant with the presence or absence of these constituents (36).

3.7. Acute toxicity testing

After being fasted for 3hrs, food but not water, five female Swiss albino mice weighing 25-27g were taken. The first mouse was given 2 g/kg crude extract (i.e.50mg for 25g mouse); the test crude extract solution was administered safely in a single dose orally by gavage. After the substance was administered, food was withheld for further 2 hours. The mouse was observed for 24hrs and it was survived so that we dosed two additional mice and both animals survived and the whole mice were observed for any signs of toxicity daily for 14 days to assess safety of the extract without dosing of further animals. Animals were observed for gross changes such as loss of appetite, hair erection, lacrimation, tremors, convulsions, salivation, diarrhea, weight change and other signs of overt toxicity (76).

3.8. Parasite inoculation

Female albino mice from the Ethiopian Health and Nutrition Research Institute (EHNRI) Addis Ababa, Ethiopia and Gondar University, Gondar, Ethiopia, previously infected with *Plasmodium berghei* (ANKA) and having parasitemia level of 20 and 25% were used as a donor; parasites are maintained by serial blood passage from mouse to mouse. Donor mice then sacrificed by head blow and blood was collected by severing the jugular vein into a tube containing 0.4% tri-sodium citrate. This was diluted with physiological saline (0.9%) based on parasitemia level of the donor mice in such a way that 1 ml blood contains 5×10^7 infected erythrocytes .Then each mouse were inoculated intraperitoneally with 0.2ml of blood suspension containing about 1×10^7 *P. berghei* parasitized erythrocytes (77, 78, 81).

3.9. Dosing and grouping of animals

Animals were randomly assigned to five Groups of each consisting of five mice. The Groups I, II and III were orally administered with crude extract and solvent fractions of 100,200 and 400mg/kg of body weight respectively. Group IV (negative control) was treated with the distilled water (chloroform and hexane fractions with 7% Tween-80 suspension) 10ml/kg (77) and Group V was administered orally with standard drug (chloroquine 25mg/kg body weight)(76).

3.10. The 4-days suppressive test

Evaluation of suppressive effect (schizontocidal activity) of the crude extract and solvent fractions on early infection against chloroquine sensitive *P. berghei* infected mice was done. And the mice were randomly grouped into five Groups of five mice and inoculated with parasite as described above (5.9.). Groups I, II, III were orally administered with the crude extract and solvent fractions at doses of 100,200 and 400mg/kg body weight, Group IV was administered orally with distilled water, while Group V was administered orally with chloroquine 25 mg/kg body weight for four consecutive days(D0-D3) and all treatments were started 3 h post-infection, then % inhibition ,parasitemia, weight change and survival time were determined (82).

3.11. Rane's test -Curative test

The curative test was undertaken with chloroform solvent fraction which showed the highest parasitemia suppression in the 4-day suppressive test. The mice were injected intraperitoneally with standard inoculum of 1x 10^7 *P. berghei* infected erythrocytes on the first day (day 0). Seventy-two hours later, the mice were divided into five groups of five mice per group as described in section 5.9 and treated accordingly. The treatments were continued once daily for 5 days starting from day 3 through day 7. On days 6, 7 and 8, Giemsa stained thin blood films were prepared from the tail of each mouse, then parasitemia level and survival time of the fraction were determined with respect to the control groups (78).

3.12. Determination of body weight and temperature changes

For the 4-day suppressive test, body weight of each mouse was measured before infection (day 0) and on (day-4) using a sensitive digital weighing balance (Item.no.E11140,Switzerland) (82) for curative treatment before infection(day0) and (day5). Likewise, rectal temperature was also measured with a digital thermometer before infection and after treatment for both test types (82).

3.13. Peripheral smear test for parasitemia

After four days of treatment, on the 5^{th} day thin blood smears were made from the tail of each mouse (83). The blood films were fixed with methanol, stained with 10 % Giemsa at pH 7.2 for 10 min, then the stained slides were washed gently using distilled water and air dried at room temperature and parasitemia were determined under Olympus microscope (CX21FS1, China) with an oil immersion nose piece of 100× magnification by counting 5-fields of approximately 200 erythrocytes per field. The difference between the mean value of the control group (negative control taken as 100%) and those of the experimental groups were calculated and expressed as percent parasitemia reduction or activity (77, 81).

$$\text{Activity} = 100 - (\text{Mean parasitemia treated}/\text{Mean parasitemia of control}) * 100$$

$$\%\ \text{Parasitemia} = (\text{No. of parasitized RBC}/\text{Total No. of RBC counted}) * 100$$

3.14 .Packed cell volume measurement (PCV)

PCV is a measure of the proportion of RBCs to plasma and measured before inoculating the parasite and after treatment with the extract .PCV measurement was done to predict the effectiveness of the test extract and fractions in preventing hemolysis due to malaria parasites. Blood was collected by heparinized capillary tubes from the tails of each mouse. The capillary tubes were filled with each mouse's blood up to 75% of their volume and sealed at the heparin end with sealing clay. The tubes were then placed in a hematocrit centrifuge (Hettich Hematokrit-210, Germany) with the sealed end outwards and centrifuged for 5 min at 10,000 rpm. The tubes were then taken out of the centrifuge and PCV was determined using a standard Micro-Hematocrit Reader (Hawksley and Sons, England) (84).

The relation is as follows;

PCV = (Volume of erythrocytes in a given volume of blood/Total blood volume)

3.15. Determination of mean survival time (MST)

Mortality was monitored daily and the number of days from the time of inoculation of the parasite up to death was recorded for each mouse in the treatment and control groups throughout the follow up period. MST for each group was calculated as follows (85).

$$\text{MST} = (\text{sum of survival days of all mice in a group}/\text{Total number of mice in that group})$$

3.16. Statistical analysis

Results of the study were presented as mean ± SEM. Data was analyzed using SPSS version 20. Statistical significance was determined by One-way ANOVA coupled to Tukey's HSD technique to compare result between doses and among treatment and control groups. For all the data obtained, the result was considered significant at 95% confidence level and P-value < 0.05.

3.17. Ethical clearance

Animal handling were according to the guide lines for Care and Use of Laboratory Animals(74, 76). Furthermore, procedures were approved by the Institutional Review Board of the University of Gondar, Department of Pharmacology

4. Results

4.1. Yield

Deep yellow semisolid 80% methanolic crude extract of *C. simensis* roots was found with the percentage yields for crude extract, n-hexane, chloroform and aqueous fractions of the plant were; 8.74%, 0.23%, 1.4% and 98.36% respectively.

4.2. Acute toxicity study

The acute toxicity study indicated that the tested three mice were survived and then no any signs of toxicity for 14 days up to 2g/kg. As a result gross physical and behavioural observation of the experimental mice revealed no visible signs of acute toxicity like lacrimation, hair erection, and reduction in their motor and feeding activities. Furthermore, they were physically active. Thus oral LD50 was found to be greater than the limit dose, 2g/kg body weight.

4.3. Phytochemical screening

The summary of the phytochemical constituents of 80%methanolic crude extract of *C. simensis* roots revealed that it showed positive reactions to alkaloids, phenolics, flavonoids and terpenoids

Table 1.Results of phytochemical screening of 80%methanolic crude extract of C. simensis roots

Phytochemicals	Crude extract	Test type	Appearance when positive
Alkaloids	+	Wagner's and Mayer's tests	Reddish brown and White creamy ppt.
Tannins	_	Braymer's test	Blue/Greenish color
Saponins	_	Foam test	Foam
Flavonoids	+	Alkaline reagent test	Colour less
Terpenoids	+	H_2SO_4(3ml)+Chloroform(2ml)	Reddish brown
Steroids	-	H_2SO_4(10ml)+Chloroform(10ml)	Brown(bottom),color less (upper)
Phenols	+	$FeCl_3$	Bluish black
Glycosides	-	Keller-Killani test	Brown ring on interface
Anthraquinones	-	Chloroform + NaOH	Red color

Note - (+) indicates presence, (-) indicates absence, PPT-Precipitate

Table 2.Body weight, temperature and PCV measurements in 4 days suppressive test of **80% methanolic crude extract** of C.simensis roots

Treatment groups(mg/kg)	Wt. before	Wt. after	Temp.before	Temp.after	PCV-before	PCV-after
Pos.control	23.6±0.3	25±0.75	36.6±0.1	36.4 ±0.2	64.8±3.4	63.8±3.6
Neg.control	25±0.30	23±0.87	36.5±0.3	34.9±0.1	63.4±2.8	58.4±2.9
100	25.9±0.9	24.7±0.56	36±0.2	35.3±0.2	60.8±3.2	56.8±2.9
200	24.9±0.8	23.5±1.2	36.3±0.2	35.4±0.2	60.6±2.7	56.8±2.6
400	26.8 ±0.8	25.4±0.6	36.7±0.2	36.1±0.1	61.0 ±3.4	57.6 ±3.3
600	26.4±0.5	25.7±0.4	36.9±0.2	36.2±0.2	58 ±5.5	55.4±5.1

Table 3. Body weight, temperature, parasitemia, survival time and PCV measurements in 4 days suppressive test of **80%methanolic crude** extract of C.simensis roots

Treatment groups(mg/kg)	Wt.diff.(g)	Temp.diff.(°C	%Parasitemia	% supp	Survival(days)	%PCV.diff
Pos.control	$1.65\pm0.68^{a}{}_{1}$	$-0.18\pm0.25^{a}{}_{3}$	$0.00\pm0.00^{\infty}{}_{3}$		$30\pm0.00^{\infty}{}_{3}$	$-1.0\pm0.45^{a}{}_{2}$
Neg.control	-1.93±0.74	-1.56±0.32	56.82±2.2	0	6.0±0.45	-5±0.1.14
100	-1.25±0.79	-0.68±0.2	57.6±5.21		6.0±0.32	-4±0.78
200	-1.4±0.55	-0.88±0.15	50.2±5.38	11.65	6.6±0.40	-3.8±0.74
400	-1.37±1.37	$-0.64\pm0.09^{a}{}_{1}$	44.6±3.4	21.5	$9.0\pm0.55\,{}^{a}{}_{1}{}^{b}{}_{1}$	-3.4 ±0.51
600	-0.64±0.11	$-0.9\pm0.16^{a}{}_{1}$	$26.4\pm2.15^{a}{}_{3}{}^{b}{}_{3}{}^{c}{}_{3}{}^{d}{}_{1}$	53.54	$10.2\pm1.07^{a}{}_{3}{}^{b}{}_{3}{}^{c}{}_{2}$	-2.6±0.6

Results are expressed as mean ±SEM, n=5, a = compared to Neg.control (dis.H_2O) b=comp.to100mg/kg, c=comp.to 200mg/kg, d=comp.to400mg/kg, e=comp.to.600mg/kg,1= $p < 0.05$, 2 = $p < 0.01$, 3= $p < 0.001$, ∞ = all treatments

Table 4.Body weight, temperature and PCV measurements in 4 days suppressive test of **aqueous fraction** of C.simensis roots

Treatment groups(mg/kg)	Wt.before	Wt. after	Temp.before	Temp.after	PCV.before	PCV.after
Pos.control	27.6±0.2	27.9±0.2	36.9±0.3	36.8±0.3	56.8±3.2	57.4±3.2
Neg.control	27.8±0.2	25.5±0.5	37.3±0.2	34.6±0.3	61.2±1.7	54.2±1.9
100	23.8±0.5	21.9±0.6	36.9±0.1	34.4±0.4	59.2±3.8	52.6±5.3
200	26.4±0.4	25.1±0.8	36.9±0.1	34.9±0.4	60.4±4.3	54±5.5
400	27.3±0.1	26.1±0.5	37±0.10	35±0.20	59.2±6.4	54.2±8.1

Table 5.Body weight, temperature, parasitemia, survival time and PCV measurements in 4 days suppressive test of **aqueous fraction** of C.simensis roots

Treatment group(mg/kg	Wt.diff.(g)	Temp.diff.(°C)	%Parasitem	%Suppn.	Survival/day	%PCV.diff
Pos.control	0.3±0.095$^{a}_{1}$	-0.10±0.18$^{a}_{2}{}^{b}_{2}{}^{c}_{1}{}^{d}_{1}$	0.00±0.00$^{\infty}_{3}$		30±0.00$^{\infty}_{3}$	0.6±0.4$^{a}_{1}$
Neg.control	-2.32±0.55	-2.7±0.38	68.4±2.71	0	5.4±0.24	-7.0±0.45
100	-1.85±0.95	-2.56±0.51	62.2±4.81	9.1	5.8±0.58	-6.6±1.8
200	-1.35±0.63	-2.00±0.48	53.5±4.17	21.8	7.6±0.51	-3.6±2.3
400	-1.27±0.4	-1.98±0.11	53.2±5.15	22.2	8.8 ±0.86$^{a}_{2}{}^{b}_{2}$	-5 ±2.51

Results are expressed as mean ±SEM, n=5, a = compared to Neg.control (dis.H_2O) b=comp.to100mg/kg, c=comp.to 200mg/kg, d=comp.to400mg/kg, 1= $p < 0.05$, 2 = $p < 0.01$, 3= $p < 0.001$, ∞ = all treatments

Table 6.Body weight, temperature and PCV measurements in 4 days suppressive test of **hexane fraction** of *C.simensis* roots

Treatment groups (mg/kg)	Wt.before	Wt.after	Temp.before	Temp.after	PCV-before	PCV-after
Pos.control	25.1±0.7	25.5±0.7	36.5±0.3	36.9±0.4	53.4±2.7	52.6±2.4
Neg.control	26.8±0.4	24.7±0.2	36.5±0.4	33.9±0.1	52±1.0	46.8±1.9
200mg/kg	25.1±0.5	23.5±0.6	37.5±0.2	35±0.2	44.2±3.4	41±3.5
400mg/kg	26.1±0.4	24.8±0.6	36.9±0.2	34.9±0.1	47.4±2.0	44.6±2.3

Table 7.Body weight, temperature, parasitemia, survival time and PCV measurements in 4 days suppressive test of **hexane fraction** of *C.simensis* roots

Treatment groups(mg/kg)	Wt.diff.(g	Temp.diff.(°C)	%Parasitemia	%Supprn.	Survival(days)	%PCV.diff
Pos.control	0.4±0.09$^{a}{}_{3}{}^{b}{}_{3}{}^{c}{}_{2}$	0.4±0.2$^{\infty}{}_{3}$	0.00±0.00$^{\infty}{}_{3}$		30±0.00$^{\infty}{}_{3}$	-0.8±0.37$^{a}{}_{2}$
Neg.control	-2.14±0.4	-2.5±0.4	44±6.4	0	6.6±0.24	-5.2±1.1
200	-1.6±0.3	-2.5±0.2	42.7±2.9	3	7.4±0.24	-3.2±0.4
400	-1.3±0.2	-2.00±0.2	40.3±2.3	8.4	7.6±0.24$^{a}{}_{1}$	-2.8±0.8

Results are expressed as mean ±SEM, n=5, a = compared to Neg.control (7% Tween-80 suspension, b=200mg/kg, 400mg/kg1= $p < 0.05$, 2= $p < 0.01$, 3= $p < 0.001$ ∞ = all treatments

Table 8.Body weight, temperature and PCV measurements in 4 days suppressive test of **chloroform fraction** of C.simensis roots

Treatment groups(mg/kg)	Wt.before	Wt.after	Temp.before	Temp.after	PCV.before	PCV.after
Pos.control	27.1±0.5	28.2±0.4	37.2±0.3	37±0.3	52±0.9	52.6±0.9
Neg.control	27.3±0.4	23.3±0.8	37.7±0.3	34.8±0.1	55±0.3	46±2
100	27.5±0.2	24±0.3	37.3±0.3	34.8±0.3	70.6±2.2	63±3.3
200	26.6±0.8	24.7±1.4	36.7±0.2	35.5±0.4	70.0±1.4	63.2±3
400	27.6±0.3	26.4±1.2	36.9±0.1	35.7±0.1	73.2±2.1	67.4±2.9

Table 9.Body weight, temperature, parasitemia, survival time and PCV measurements in 4 days suppressive test of **chloroform fraction** of C.simensis roots

Treatment groups(mg/kg)	Wt.diff.(g)	Temp.diff.(°C)	%Parasitemia	%Suppression	Survival(day)	%PCV.diff
Pos.control	1.1±0.09$^{a}_{1}$	-0.14±0.098$^{a}_{3}{}^{b}_{2}$	0.00±0.00$^{\infty}_{3}$		30±0.00$^{\infty}_{3}$	0.6±0.24$^{a}_{2}$
Neg.control	-4.0±0.5	-2.94±0.35	72.6±1.36	0	5.0±0.32	-9.0±1.8
100	-3.43±0.26	-2.5±0.5	71.0±4.04	2.2	6.2±0.49	-7.6±1.3
200	-1.9±2.15	-1.28±0.5$^{a}_{1}$	59±5.41	18.73	6.8±0.58	-6.8±1.9
400	-1.22±1.33	-1.2±0.18$^{a}_{1}$	53.4±3.6$^{a}_{2}{}^{b}_{1}$	26.45	7.2±0.86$^{a}_{3}{}^{b}_{1}$	-5.8 ±1.16

Results are expressed as mean ±SEM, n=5, a = compared to Neg.control (7% Tween-80 suspension) b=comp.to100mg/kg, c=comp.to 200m/kg, d=comp.to400mg/kg, 1= $p < 0.05$, 2 = $p < 0.01$, 3= $p < 0.001$, ∞ = all treatments

Table 10.Tempreature, parasitemia and survival time measurements for **Rane's** test of **chloroform fraction** of C.simensis roots

Treatment group(mg/kg)	Temp.befor e	Temp.after	Para-day 6	Para-day 7	Para. day 8	% Inhi bn	Survival (day)
Pos.control	37.2±0.3	37.1±0.3	$3\pm0.16^{\infty}{}_{\mathbf{3}}$	$1.02\pm0.27^{\infty}{}_{\mathbf{3}}$	$0.00\pm0.00^{\infty}{}_{3}$		$30\pm0.000^{\infty}{}_{3}$
Neg.control	37.7±0.3	34.8±0.1	21±1.14	22.8±0.97	23.4±0.75	0	5.6±0.24
200	37.3±0.3	34.8±0.3	19.8±0.97	22 ±1.05	23.0±1.05	1.71	6.6±0.24
400	36.7±0.2	35.5±0.4	17.6±1.89	$18.4\pm1.4^{a}{}_{1}$	$19.0\pm1.18^{\mathbf{a_1b_1}}$	18.8	$8.4\pm0.4^{a}{}_{3}{}^{b}{}_{2}$
600	36.9±0.1	35.7±0.1	$13.8\pm0.73^{\mathbf{a_2b_1}}$	$10.2\pm0.66^{a}{}_{3}{}^{b}{}_{3}{}^{c}{}_{3}$	$9.4\pm0.68^{a}{}_{3}{}^{b}{}_{3}{}^{c}{}_{3}$	59.8	$10.8\pm0.37^{a}{}_{3}{}^{b}{}_{3}{}^{c}{}_{3}$

Results are expressed as mean ±SEM, n=5, a = compared to Neg.control (7% Tween-80 suspension), b=comp.to 200mg/kg, c=comp.to 400mg/ kg d=comp.to 600mg/kg, 1= $p < 0.05$, 2 = $p < 0.01$, 3= $p < 0.001$, ∞ = all treatments, Para =parasitemia

Table 11.Rectal temperature, weight and PCV measurements in Rane's test of **chloroform fraction** of C.simensis roots

Treatment grps.(mg/kg)	Wt.befor	Wt.after	PCV-befor	PCVafter	Wt.diff.	Temp.diff	PCV.diff
Pos.control	27.1±0.5	27.4±0.5	52±0.9	52.6±0.9	$0.5\pm0.07^{\infty}{}_{\mathbf{3}}$	$0.06\pm0.07^{\infty}{}_{\mathbf{3}}$	$-1.52\pm0.22^{a}{}_{3}{}^{b}{}_{2}$
Neg.control	27.3±0.4	24.1±0.1	55±0.3	50.4±0.7	-4.1±0.23	-8.13±0.49	-15.5±2.5
200	27.5±0.2	24±0.3	70.6±2.2	58.6±6.5	-3.39±0.44	-7.4±0.53	-10.3±2.2
400	26.6±0.8	24.7±1.4	70±1.4	61.6±6.2	$-2.58\pm0.17^{a}{}_{2}$	$-6.7\pm0.32^{\mathbf{a_2}}$	$-7.3\pm0.54^{\mathbf{a_2}}$
600	27.6±0.3	26.4±1.2	73.2±2.1	67.4±2.9	$-1.9\pm0.25^{a}{}_{3}{}^{b}{}_{2}$	$-4.98\pm0.32^{\mathbf{a_3b_2}}$	$-5.1\pm0.32^{\mathbf{a_2}}$

Results are expressed as mean ±SEM, n=5, a = compared to Negative control (7% Tween-80 suspension) b=comp.to 200mg/kg, c=comp.to 400mg/kg, d=comp.to 600mg/kg, 1= $p < 0.05$, 2 = $p < 0.01$, 3= $p < 0.001$, ∞ = all treatments, Positive control=Chloroquine -25mg/kg.

5. Discussion

No single solvent has the ability to extract and dissolve all the plant metabolites at once, rather several solvents of increasing polarity need to be used successively, such as hexane → ethyl-acetate →chloroform → methanol → ethanol → water (39).Therefore, for our extraction we used methanol (for crude), n-hexane, chloroform and distilled water in order not to miss the polar and non-polar plant metabolites within the roots.

Even though Ethiopian traditional medicine practitioners applied squeezing and homogenization techniques for the extraction of bioactive components from *C. simensis* roots for the treatment of malaria, for our study we used 80% aqueous methanol using maceration technique as it is the most effective solvent extracting both more polar and moderately polar plant constituents most efficiently from plant parts - alkaloids, flavonoids, phenolics etc (86-88).

The yield of 80% methanolic crude extract was deep yellow semisolid mass, 166g (8.74%), from which we used 151g for fractionation: aqueous fraction 148.53g (98.37%), chloroform fraction 2.13g (1.4%) and n-hexane 0.35g (0.23%). The least yield being the n-hexane fraction in consistent with a research on the leaves of *Flueggea virosa*-2.6% (89).And the activity resides on polar components. The probable reason for this great yield variation might be due to high concentration of polar compounds in the root of the plant species that better dissolve in water. But from a research report on the leaf of the same plant the yield was deep green semisolid and their percentages were; petroleum ether-13.5%, chloroform-5%, acetone-8.3%, methano-11.7%, the highest yield being in the non polar component in disagreement with our findings, however ,the anti-inflammatory and anti-nociceptive activities still reside on the polar part(in acetone and methanol) (90).

Changes in general behaviours, variations in body weight and morbidity are critical for the evaluation of the effect of a compound on test animals, since such changes are often the first signs of toxicity (76).Therefore this crude extract was tested for its toxicity against female Swiss albino mice. None of the test mice died or showed no signs of acute toxicity within 24 hours of treatment and then 14 days of follow up, up to 2g/kg, however, it doesn't mean that it is safe in long term use. As a result its LD50 was assumed to be more than 2g/kg body weight.

Although chloroquine is not a first-line drug in the treatment of malaria in humans, it is used as control drug in this study as the *P.berghei* used for inoculation in this study is a chloroquine-sensitive strain (77).When a standard antimalarial drug, 25mg/kg Chloroquine was used for treatment of mice infected with *P. berghe*i (76), it clears parasitemia to undetectable levels, 0% in both crude and solvent fractions.

Due to reasonably high homology and similarity between mammalian genetics and physiology and to the relatively short reproductive cycle, mouse models are the most used for assessment of antiplasmodial activities of plant extracts and products derived from them and hence for our study we used female Swiss albino mice of age 8-9 weeks (39). In this age range, many developmental processes are going on and changes in physiology with age will have a large impact on experimental variables. For instance, B-cells have an immature phenotype until four weeks of age, T-cell responses mature around eight weeks of age, and T and B-lymphocyte production increases over the first 26 weeks of life. Development of mouse spinal cord, hippocampus and olfactory structures is ongoing until 11 weeks of age. In addition older animals entering senescence may respond differently to their younger counterparts (91).

In vivo evaluation of antimalarial effects of our extract was done with the use of rodent malaria parasite, *P. berghe*i as *Plasmodium* species that cause human disease are essentially unable to infect non-primate animal models (with the exception of a complex immunocompromised mouse model that has been developed to sustain *P. falciparum*-parasitized human erythrocytes *in vivo*) and rodent models have been validated through the identification of several antimalarials - for instance, mefloquine, halofantrine and artemisinin derivatives(77) and also it is cost effective preliminary pharmacological activity screening.

In this study a four-day suppressive test - to evaluate antimalarial activities of extract on early infection and curative tests-to evaluate curative potential on established infections were used by comparison of blood parasitemia, PCV, weight change, temperature reduction and survival time in treated and untreated mice.

Anemia, rectal temperature reduction, and reduction in body weight are the general features of malarial infection in mice and human malarial infections (92, 93). Therefore a potent

antimalarial is expected to improve anemia, prevent body weight loss, and stabilize temperature in infected mice with increasing parasitemia.

From a research, anti-malarial activities of Maslinic acid (MA), a natural triterpene, which showed a parasitostatic action in-vitro on *P. falciparum*, analysed in vivo on international center for research-ICR mice using the *P. yoelii* 17XL murine model (Py17XL), the survival increased from 20 to 80% after four-day suppressive MA test with a dose of 40 mg Kg/day on Py17XL infected mice with a parasitemia profile similar to that of the untreated mice. That was due to the parasitostatic effect exerted by MA during the first days of infection (94).

The same analogy might be applied here, our crude extract's parasitemia reduction in a four-day suppressive test was not statistically significant but % suppression was 21.5% (i.e. mean parasitemia level=78.5%) for 400mg/kg compared with negative control and mean group parasitemia level of ≤ 90% of that of treated versus negative control animals usually indicates that the test compound is active in standard screening studies(95, 96), in addition, this dose brought significant survival benefit in comparison with 100mg/kg ($P < 0.05$) and negative control ($P<0.05$) this might be attributed from parasitostatic effect of our extract having terpenoid component or so in consistent with the above report , and that might favour the development of more effective sterilizing immune responses. In the same manner 400mg/kg aqueous fraction showed survival advantage in a four-day suppressive test in comparison with 100mg/kg ($P<0.01$) and negative control ($P<0.01$)but this dose brought no statistically significant parasitemia reduction(% suppression=22.5), this might be due to the fact that the aqueous fraction has parasitostatic effect.

Additionally our crude extract's % suppression was 53.54 (i.e. mean parasitemia level = 46.46%) and parasitemia reduction was statistically significant for 600mg/kg compared with negative control, 100mg/kg and 200mg/kg ($P<0.001$), with 400mg/kg ($P<0.05$) and. This same dose gave significant survival advantage in relation with negative control and 100mg/kg ($P< 0.001$), with 200mg/kg ($P<0.01$).(Table3) Due to these findings and the above reports we passed on to fractions screening as the "Thompson Survival Assay" and the "Peters' 4-day tests" are the most widely used efficacy assays in malaria (77, 97)

PCV was measured in this study to determine the effectiveness of both crude extract and solvent fractions (i.e., aqueous, chloroform and hexane fractions) of *C.simensis root* in preventing malaria-induced hemolysis. Saponins having amphiphilic properties can interfere with biological surfaces such as cell membranes-including erythrocyte membranes. This may result in haemolysis as a consequence of perturbation and loss of the cell membrane integrity (which are non-specific and may affect wide range of organisms (98)) but with unknown mechanism (93).In addition, saponins reduce protein digestibility by formation of saponin-protein complexes and obstruct the absorption of micronutrients (98). In our extract no saponins were found, therefore that insignificant hemolysis observed in all treated and control groups in Peters' 4-day tests were due to the parasitemia level only.

There was reduction of rectal temperature with increasing parasitemia in the infected mice, in all the crude and fractions' doses administered but the positive control didn't. The reduced rectal temperature might be attributed to reduced metabolic activities due to the progressive infection and accompanying appetite.

From amongst the treatment groups the highest dose 600 and 400mg/kg crude extract showed comparable significant temperature stabilizing effect ($P<0.05$) and 400mg/kg chloroform fraction also significantly controlled the temperature reduction ($P<0.05$) followed by 200mg/kg of the same fraction ($P<0.05$) in relation to the negative control but all other treatments did not stabilize temperature in Peter's test. (Table3)

The 400mg/kg chloroform fraction also showed significant parasitemia reduction ($p<0.01$), ($p<0.05$) and survival benefit ($p<0.001$),($p<0.05$)when compaired with negative control and 100mg/kg, respectively. The fact that 400mg/kg chloroform fraction showed the highest reduction of parasites but all other treatment groups (crude and fractions) showed no significant parasitemia reduction by this dose; suggests that the active ingredients of this plant responsible for its antimalarial activities may be localized in this fraction, like the research conducted on *C. macrostachyus* (78). In addition % suppression of this dose was 26.45% in 4-days Peter's test when compared with negative control (Table9).

Insignificant weight loss was recorded in all the doses of crude extract and solvent fractions of *C. simensis* administered, but there was significant weight reduction in negative control in relation

to positive one. This result shows that, the extract has controlled the weight lose that would otherwise be expected in malaria infection. This change might be solely due to reduced feed intake by the influence of the parasite itself as the extracts are negative for appetite suppressing components like saponins, glycosides and tannins (92, 99). This suppression might be due to the precipitation of proteins and gastrointestinal enzymes thereby reducing the digestibility and availability of proteins (98). The n-hexane fraction brought no significant advantage, except survival benefit when we evaluated it by using the five parameters mentioned above.

In this study we have also conducted Rane's test; after the last day of treatment parasitemia and survival advantage were found to be significant for 400mg/kg ($P<0.05$) and ($P<0.001$, $P<0.01$) compared with negative control and 200mg/kg respectively, for 600mg/kg compared with negative control, 200mg/kg and 400mg/kg ($P<0.001$) dose dependently. In addition survival time to more than twice that of the negative controls is considered as evidence of activity (31). Correspondingly, significant control of weight and temperature reduction were observed in relation with negative control, for 400mg/kg ($P<0.01$), for 600mg/kg ($P<0.001$), additionally for 600mg/kg in relation with 200mg/kg ($P<0.01$). PCV was significant too for 600 and 400mg/kg ($P<0.01$) in comparison with negative control (Table11).

The chemotaxonomy of the genes *Clematis* is carried out using flavonoids, triterpenoid saponins, ribose, etc profiles as chemical markers for the taxonomy of the Ranunculaceae (47, 49). Our study showed that *C. simensis* root has the following secondary metabolites which have been suggested to be responsible for antiplasmodial activity of other plants; alkaloids, flavonoids, terpenoids and phenols in agreement with the above report but saponins. Therefore, the observed antimalarial activity could be associated with some of the alkaloids and other constituents reported from the plant in synergy (100) , as alkaloids such as quinine (98), sesquiterpene trioxane lactone (Artemisinin) (101) and flavonoids (100) are known for their antimalarial activities. Also chemo-taxonomical survey revealed that alkaloids are widely distributed in higher plants like Ranunculaceae (98), this is in agreement with our finding in that we observed alkaloid in both Wagner's and Mayer's tests during phytochemical screening.

Oxidative stress is observed in the malaria development. Plasmodia digest haemoglobin which results in the production of heme and heme triggers the production of reactive oxygen SPP. which are implicated in the pathophysiology of malaria and can lead to anemia and apoptosis. Plants having a high concentration of phenolic compounds like phenol itself and flavonoids (poly-phenols) are a reservoir of novel antioxidant molecules (102). A study showed the hepatoprotective (antioxidant) effect of *C. chinensis* extract on CCL4-induced hepatotoxicity in rats and the results of aminetransferase, aspartate aminetransferase and alanine aminetransferase , oxidant defence enzymes , have shown a significant hepatoprotective effect after treatment with *C. Chinensis (52).* In our study we have found phenolic metabolites too, and the observed antimalarial effect may come from these and other metabolites screened.

Ethno pharmacologically different parts of *C. Simensis* are reported to be a remedy for analgesia, and malaria (68, 70) , malaria and liver disease (71) and anti-inflammatory and antibacterial in humans (54) as inflammation is one of the complications of malaria parasite infection and drugs having antibacterial activity such as tetracycline and its derivatives have been used in malarial treatment. Various Clematis species have been shown experimentally to exhibit significant anti inflammatory and analgesic activities (47, 49). Methanolic extract of *C.campestris;* flower, showed good *in vitro* antiplasmodial activity against *P. falciparum* (59).

Our evaluation of the plant against a strain of *P.berghei in vivo* proved significant antimalarial activities with no toxicity up to 2g/kg, in agreement with another study on in vivo anti inflammatory and anti-nociceptive activities of the leaf extracts of the same plant having no toxicity from 2-5mg/kg(90) .

The results of this study were therefore, in consistent with the traditional use of this plant for antimalarial therapy. Therefore, this study together with the aforementioned reports showed that the plant may serve as a potential source of different antiplasmodial compounds.

6. Limitation of the Study

We haven’t done sub acute toxicity and quantitative phytochemical tests and these limit the importance of this study.

7. Conclusion

The hydroalcholic crude extract and chloroform fraction showed significant antimalarial activities and the present study has demonstrated that the antimalarial activity of the plant was significantly improved through fractionation of the methanolic crude extract of its roots. These results suggest that the Kunama and Gumuz ethnic groups of Ethiopia antimalarial application of *C. simensis* have a pharmacological basis. These results suggest that it is possible to isolate active anti-malarial compounds from the extract as well as to determine their mechanism(s) of action.

8. Recommendation

From our study, the following works are suggested for further investigation on the plant; further phytochemical investigation to clearly identify and quantify the antiplasmodial active components from the plant, sub acute and chronic toxicity study to know the safety of *C. simensis roots* and investigation of in-vivo antimalarial activities against non *P.berghei* spp. in order to consider them as potential sources for antimalarial drug development for human malaria.

9. References

1. Srivastava A, Creek DJ, Evans KJ, De Souza D, Schofield L, Müller S, et al. Host reticulocytes provide metabolic reservoirs that can be exploited by malaria parasites. PLoS Pathog. 2015;11(6):e1004882.

2. Hanboonkunupakarn B, White NJ. The threat of antimalarial drug resistance. Tropical Diseases, Travel Medicine and Vaccines. 2016;2(1):10.

3. Crompton PD, Moebius J, Portugal S, Waisberg M, Hart G, Garver LS, et al. Malaria immunity in man and mosquito: insights into unsolved mysteries of a deadly infectious disease. Annual review of immunology. 2014;32:157.

4. Mboowa G. Genetics of sub-Saharan African human population: implications for HIV/AIDS, tuberculosis, and malaria. International journal of evolutionary biology. 2014.

5. Moss WJ, Dorsey G, Mueller I, Laufer MK, Krogstad DJ, Vinetz JM, et al. Malaria Epidemiology and Control Within the International Centers of Excellence for Malaria Research. The American journal of tropical medicine and hygiene. 2015;93(3 Suppl):5-15.

6. Pulice G, Pelaz S, Matías-Hernández L. Molecular Farming in Artemisia annua, a Promising Approach to Improve Anti-malarial Drug Production. Frontiers in plant science. 2016;7.

7. Cox J, Sovannaroth S, Soley LD, Ngor P, Mellor S, Roca-Feltrer A. Novel approaches to risk stratification to support malaria elimination: an example from Cambodia. Malaria journal. 2014;13(1):1.

8. WHO. Global Malaria Programe,World malaria report.2014.

9. Shelton JM, Corran P, Risley P, Silva N, Hubbart C, Jeffreys A, et al. Genetic determinants of anti-malarial acquired immunity in a large multi-centre study. Malaria journal. 2015;14(1):1.

10. Schicker RS, Hiruy N, Melak B, Gelaye W, Bezabih B, Stephenson R, et al. A venue-based survey of malaria, anemia and mobility patterns among migrant farm workers in Amhara region, Ethiopia. PloS one. 2015;10(11):e0143829.

11. Kar NP, Kumar A, Singh OP, Carlton JM, Nanda N. A review of malaria transmission dynamics in forest ecosystems. Parasites & vectors. 2014;7(1):1.

12. Kirinyet R, Ng'etich AS, Juma A. Assessment of Malaria Reporting and Epidemic Preparedness Systems in Health Facilities in Eldoret West District, Uasin Gishu County-Kenya. Journal of Public Health in Africa. 2016;7(1).

13. Demissie Y, Ketema T. Complicated malaria symptoms associated with Plasmodium vivax among patients visiting health facilities in Mendi town, Northwest Ethiopia. BMC infectious diseases. 2016;16(1):436.

14. Aregawi M, Lynch M, Bekele W, Kebede H, Jima D, Taffese HS, et al. Time series analysis of trends in malaria cases and deaths at hospitals and the effect of antimalarial interventions, 2001–2011, Ethiopia. PLoS One. 2014;9(11):e106359.

15. Nega D, Dana D, Tefera T, Eshetu T. Prevalence and predictors of asymptomatic malaria parasitemia among pregnant women in the rural surroundings of Arbaminch Town, South Ethiopia. PloS one. 2015;10(4):e0123630.

16. Sack BK, Keitany GJ, Vaughan AM, Miller JL, Wang R, Kappe SH. Mechanisms of stage-transcending protection following immunization of mice with late liver stage-arresting genetically attenuated malaria parasites. PLoS Pathog. 2015;11(5):e1004855.

17. Kaushansky A, Mikolajczak SA, Vignali M, Kappe SH. Of men in mice: the success and promise of humanized mouse models for human malaria parasite infections. Cellular microbiology. 2014;16(5):602-11.

18. WHO. Guidelines for the treatment of malaria. 3rd- ed ;2015.

19. Cullen KA. Malaria Surveillance—United States, 2013. MMWR Surveillance Summaries. 2016;65.

20. Khan SM, Reece SE, Waters AP, Janse CJ, Kaczanowski S. Why are male malaria parasites in such a rush? Sex-specific evolution and host–parasite interactions. Evolution, medicine, and public health. 2013;2013(1):3-13.

21. Fairhurst RM, Bess CD, Krause MA. Abnormal PfEMP1/knob display on Plasmodium falciparum-infected erythrocytes containing hemoglobin variants: fresh insights into malaria pathogenesis and protection. Microbes and infection. 2012;14(10):851-62.

22. Klein E. Antimalarial drug resistance: a review of the biology and strategies to delay emergence and spread. International journal of antimicrobial agents. 2013;41(4):311-7.

23. Pimenta PF, Orfano AS, Bahia AC, Duarte AP, Ríos-Velásquez CM, Melo FF, et al. An overview of malaria transmission from the perspective of Amazon Anopheles vectors. Memórias do Instituto Oswaldo Cruz. 2015;110(1):23-47.

24. Wassmer SC, Taylor TE, Rathod PK, Mishra SK, Mohanty S, Arevalo-Herrera M, et al. Investigating the pathogenesis of severe malaria: a multidisciplinary and cross-geographical approach. The American journal of tropical medicine and hygiene. 2015;93(3 Suppl):42-56.

25. Mekonnen SK, Aseffa A, Medhin G, Berhe N, Velavan TP. Re-evaluation of microscopy confirmed Plasmodium falciparum and Plasmodium vivax malaria by nested PCR detection in southern Ethiopia. Malaria journal. 2014;13(1):1.

26. Chan J-A, Fowkes FJ, Beeson JG. Surface antigens of Plasmodium falciparum-infected erythrocytes as immune targets and malaria vaccine candidates. Cellular and Molecular Life Sciences. 2014;71(19):3633-57.

27. Siciliano G, Alano P. Enlightening the malaria parasite life cycle: bioluminescent Plasmodium in fundamental and applied research. Frontiers in microbiology. 2014;6:391-.

28. Fougère A, Jackson AP, Bechtsi DP, Braks JA, Annoura T, Fonager J, et al. Variant exported blood-stage proteins encoded by Plasmodium multigene families are expressed in liver stages where they are exported into the parasitophorous vacuole. PLoS pathogens. 2016;12(11):e1005917.

29. Molla E. Malaria: What are the Needs for Diagnosis, Treatment and Control? Biology and Medicine. 2016;8(6):1.

30. WHO. World malaria report. 2016.

31. Peters W. Antimalarial Drugs I: Biological Background, Experimental Methods, and Drug Resistance: Springer; 2013.

32. CDC. Treatment of Malaria (Guidelines For Clinicians)2013.

33. Singh S, Singh R. Herbal medicinal treatment of Malaria in Aliero Local Government Area, Kebbi, Nigeria. Journal of Medicinal Plants. 2014;2(2).

34. Anywar G, van't Klooster CI, Byamukama R, Wilcox M, Nalumansi PA, de Jong J, et al. Medicinal Plants Used in the Treatment and Prevention of Malaria in Cegere Sub-County, Northern Uganda. Ethnobotany Research and Applications. 2016;14:505-16.

35. Mulata H, Gnanasekaran N, Melaku U, Daniel S. Phytochemical screening and assessment of in vitro antioxidant activities of Calpurnia aurea seeds and leaves. Ijppr Human. 2015;2(2):1-12.

36. Islam M. Phyttochemiicall and biiollogiicall Investtiigattiion off Leaff off Musa sapiienttum (Musaceae): East West University; 2012.

37. Kipkore W, Wanjohi B, Rono H, Kigen G. A study of the medicinal plants used by the Marakwet Community in Kenya. Journal of ethnobiology and ethnomedicine. 2014;10(1):1.

38. Al-Aamri KK, Hossain MA. New prenylated flavonoids from the leaves of Dodonea viscosa native to the Sultanate of Oman. Pacific Science Review A: Natural Science and Engineering. 2016.

39. Tarkang PA, Appiah-Opong R, Ofori MF, Ayong LS, Nyarko AK. Application of multi-target phytotherapeutic concept in malaria drug discovery: a systems biology approach in biomarker identification. Biomarker Research. 2016;4(1):25.

40. Megersa M, Asfaw Z, Kelbessa E, Beyene A, Woldeab B. An ethnobotanical study of medicinal plants in Wayu Tuka district, east Welega zone of oromia regional state, West Ethiopia. Journal of ethnobiology and ethnomedicine. 2013;9(1):1.

41. Gabryszewski SJ, Modchang C, Musset L, Chookajorn T, Fidock DA. Combinatorial genetic modeling of pfcrt-mediated drug resistance evolution in Plasmodium falciparum. Molecular biology and evolution. 2016;33(6):1554-70.

42. Riese P, Trittel S, Schulze K, Guzmán CA. Rodents as pre-clinical models for predicting vaccine performance in humans. Expert review of vaccines. 2015;14(9):1213-25.

43. Hoffman SL, Vekemans J, Richie TL, Duffy PE. The march toward malaria vaccines. American journal of preventive medicine. 2015;49(6):S319-S33.

44. Miura K. Progress and prospects for blood-stage malaria vaccines. Expert review of vaccines. 2016;15(6):765-81.

45. Rts S. Efficacy and safety of the RTS, S/AS01 malaria vaccine during 18 months after vaccination: a phase 3 randomized, controlled trial in children and young infants at 11 African sites. PLoS medicine. 2014;11(7):e1001685.

46. Frame I, Deniskin R, Arora A, Akabas MH. Purine import into malaria parasites as a target for antimalarial drug development. Annals of the New York Academy of Sciences. 2015;1342(1):19-28.

47. Sun F, Cai Z, Chaudhary MI, Xiao P, Cheng Y. Distribution of the triterpenoid saponins and chemotaxonomy of the genus Clematis L. by high-performance liquid chromatography–mass spectrometry. Biochemical Systematics and Ecology. 2010;38(5):1018-25.

48. Al-Taweel AM, El-Deeb KS, Abdel-Kader MS, Mossa JS. GC/MS Analysis of the Fatty Acids of Three Clematis species Growing in Saudi Arabia and their Anti-inflammatory activity. Saudi Pharmaceutical Journal. 2007;15(3-4):224-7.

49. Asres K. In vivo anti-inflammatory and antinociceptive activities of the leaf extracts of Clematis simensis Fresen. 2009.

50. Hai W, Cheng H, Zhao M, Wang Y, Hong L, Tang H, et al. Two new cytotoxic triterpenoid saponins from the roots of Clematis argentilucida. Fitoterapia. 2012;83(4):759-64.

51. Al-Taweel AM, El-Deeb KS, Abdel-Kader MS. GC/MS Analyses, Anti-Inflammatory and Anti-Microbial Activities of the Volatile Constituents of Three Clematis Species Growing in Saudi Arabia. Saudi Pharmaceutical Journal. 2008;16(2):170.

52. Jin F. The pharmaceutical potential of compounds from Tasmanian Clematis species: University of Tasmania; 2012.

53. Buzzini P, Pieroni A. Antimicrobial activity of extracts of Clematis vitalba towards pathogenic yeast and yeast-like microorganisms. Fitoterapia. 2003;74(4):397-400.

54. Ding Q, Yang L-X, Yang H-W, Jiang C, Wang Y-F, Wang S. Cytotoxic and antibacterial triterpenoids derivatives from Clematis ganpiniana. Journal of ethnopharmacology. 2009;126(3):382-5.

55. Brandenburg WA. Meclatis in Clematis: yellow flowering Clematis species: systematic studies in Clematis L.(Ranunculaceae), inclusive of cultonomic aspects: sn]; 2000.

56. Pennacchio M, Jefferson L, Havens K. Uses and abuses of plant-derived smoke: Its ethnobotany as hallucinogen, perfume, incense, and medicine: Oxford University Press; 2010.

57. Williams C. Medicinal plants in Australia volume 4: an Antipodean apothecary: Rosenberg Publishing; 2013.

58. Chawla R, Kumar S, Sharma A. The genus Clematis (Ranunculaceae): Chemical and pharmacological perspectives. Journal of ethnopharmacology. 2012;143(1):116-50.

59. Calderón AI, Simithy-Williams J, Gupta MP. Antimalarial natural products drug discovery in Panama. Pharmaceutical biology. 2012;50(1):61-71.

60. Essig FB. A preliminary analysis of Clematis (Ranunculaceae) in sub-Saharan Africa. SIDA, Contributions to Botany. 2004:861-77.

61. Al-Taweel AM. Phytochemical and Biological Studies of Some Clematis Species Growing in Saudi Arabia: King Saud University; 2007.

62. Adal H, Asfaw Z, Woldu Z, Demissew S, Damme P. An iconic traditional apiculture of park fringe communities of Borena Sayint National Park, north eastern Ethiopia. Journal of ethnobiology and ethnomedicine. 2015;11(1):1.

63. Chifundera K. Contribution to the inventory of medicinal plants from the Bushi area, South Kivu Province, Democratic Republic of Congo. Fitoterapia. 2001;72(4):351-68.

64. Enyew A, Asfaw Z, Kelbessa E, Nagappan R. Ethnobotanical study of traditional medicinal plants in and around Fiche District, central Ethiopia. Current Research Journal of Biological Sciences. 2014;6(4):154-67.

65. Getaneh S, Girma Z. An ethnobotanical study of medicinal plants in Debre Libanos Wereda, Central Ethiopia. African Journal of Plant Science. 2014;8(7):366-79.

66. Seshathri K, Thiyagarajan T. Antimicrobial Activity of Chewing Sticks of Jimma â€“Ethiopia against. Journal of Phytology. 2011;3(8).

67. Kigen G, Some F, Kibosia J, Rono H, Kiprop E, Wanjohi B, et al. Ethnomedicinal Plants Traditionally Used by the Keiyo Community in Elgeyo Marakwet County, Kenya. Journal of Biodiversity, Bioprospecting and Development. 2014;2014.

68. Cousins D, Huffman MA. Medicinal properties in the diet of gorillas: an ethno-pharmacological evaluation. 2002.

69. Meragiaw M, Asfaw Z, Argaw M. The status of ethnobotanical knowledge of medicinal plants and the impacts of resettlement in Delanta, northwestern Wello, northern Ethiopia. Evidence-Based Complementary and Alternative Medicine. 2016.

70. Gidey M, Beyene T, Signorini MA, Bruschi P, Yirga G. Traditional medicinal plants used by Kunama ethnic group in Northern Ethiopia. Journal of Medicinal Plants Research. 2015;9(15):494-509.

71. Asnake S, Teklehaymanot T, Hymete A, Erko B, Giday M. Antimalarial medicinal plants used by Gumuz people of Mandura Woreda, Benishangul-Gumuz Regional State, Ethiopia. 2016.

72. Kasa D, Woldeab G. Evaluation of Different Botanical Plant Extracts and Other Material against Enset Bacterial Wilt (Xanthomonas campestris PV Musacearum) Disease in Oromia Regional State, Ethiopia. 2011.

73. Hansen FK, Sumanadasa SD, Stenzel K, Duffy S, Meister S, Marek L, et al. Discovery of HDAC inhibitors with potent activity against multiple malaria parasite life cycle stages. European journal of medicinal chemistry. 2014;82:204-13.

74. Care IoLARCo, Animals UoL, Resources NIoHDoR. Guide for the care and use of laboratory animals: National Academies; 1985.

75. Care JWGoV. Guidelines for the veterinary care of laboratory animals: report of the FELASA/ECLAM/ESLAV Joint Working Group on Veterinary Care. Laboratory animals. 2008.

76. Oecd. OECD Guidelines for the Testing of Chemicals: Organization for Economic,425; 2008.

77. Fidock DA, Rosenthal PJ, Croft SL, Brun R, Nwaka S. Antimalarial drug discovery: efficacy models for compound screening. Nature Reviews Drug Discovery. 2004;3(6):509-20.

78. Bantie L, Assefa S, Teklehaimanot T, Engidawork E. In vivo antimalarial activity of the crude leaf extract and solvent fractions of Croton macrostachyus Hocsht.(Euphorbiaceae) against Plasmodium berghei in mice. BMC complementary and alternative medicine. 2014;14(1):1.

79. Sadiq A, Ahmad S, Ali R, Ahmad F, Ahmad S, Zeb A, et al. Antibacterial and antifungal potentials of the solvents extracts from Eryngium caeruleum, Notholirion thomsonianum and Allium consanguineum. BMC Complementary and Alternative Medicine. 2016;16(1):478.

80. Esmaeilzadeh Kenari R, Mohsenzadeh F, Amiri ZR. Antioxidant activity and total phenolic compounds of Dezful sesame cake extracts obtained by classical and ultrasound-assisted extraction methods. Food science & nutrition. 2014;2(4):426-35.

81. Kalra BS, Chawla S, Gupta P, Valecha N. Screening of antimalarial drugs: An overview. Indian journal of pharmacology. 2006;38(1):5.

82. Peters W, Portus J, Robinson B. The four-day suppressive in vivo antimalarial test. Ann Trop Med Parasitol. 1975;69:155-71.

83. Mishra PS, Sharma H, Mishra R, Gupta S. A Review on Anti-malarial Drug Discovery and its Screening Method. 2014.

84. Girma S, Giday M, Erko B, Mamo H. Effect of crude leaf extract of Osyris quadripartita on Plasmodium berghei in Swiss albino mice. BMC complementary and alternative medicine. 2015;15(1):184.

85. Lawal B, Shittu OK, Kabiru AY, Jigam AA, Umar MB, Berinyuy EB, et al. Potential antimalarials from African natural products: A reviw. Journal of intercultural ethnopharmacology. 2015;4(4):318.

86. Ji H, Fannin F, Klotz J, Bush L. Tall fescue seed extraction and partial purification of ergot alkaloids. Frontiers in chemistry. 2014;2.

87. Anwar F, Qayyum HMA, Hussain AI, Iqbal S. Antioxidant activity of 100% and 80% methanol extracts from barley seeds (Hordeum vulgare L.): stabilization of sunflower oil. Grasas y aceites. 2010;61(3):237-43.

88. Ashraf A, Bhatti IA, Sultana B, Jamil A. Study of Variations in the Extraction Yield, Phenolic Contents and Antioxidant Activities of the Bark of F. religiosa as a Function of Extraction Procedure. Journal of Basic and Applied Sciences. 2016;12:8-13.

89. Singh SV, Manhas A, Kumar Y, Mishra S, Shanker K, Khan F, et al. Antimalarial activity and safety assessment of Flueggea virosa leaves and its major constituent with special emphasis on their mode of action. Biomedicine & Pharmacotherapy. 2017;89:761-71.

90. Tadele A, Asres K, Melaku D, Mekonnen W. In vivo anti-inflammatory and antinociceptive activities of the leaf extracts of Clematis simensis Fresen. Ethiop Pharm J. 2009;27:33-41.

91. Jackson SJ, Andrews N, Ball D, Bellantuono I, Gray J, Hachoumi L, et al. Does age matter? The impact of rodent age on study outcomes. Laboratory animals. 2017;51(2):160-9.

92. Böttger S, Melzig MF. The influence of saponins on cell membrane cholesterol. Bioorganic & medicinal chemistry. 2013;21(22):7118-24.

93. Böttger S, Hofmann K, Melzig MF. Saponins can perturb biologic membranes and reduce the surface tension of aqueous solutions: a correlation? Bioorganic & medicinal chemistry. 2012;20(9):2822-8.

94. Moneriz C, Marín-García P, Bautista JM, Diez A, Puyet A. Parasitostatic effect of maslinic acid. II. Survival increase and immune protection in lethal Plasmodium yoelii-infected mice. Malaria journal. 2011;10(1):103.

95. Nafiu MO, Abdulsalam TA, Akanji MA. Phytochemical analysis and antimalarial activity aqueous extract of Lecaniodiscus cupanioides root. Journal of tropical medicine. 2013.

96. Tarkang PA, Okalebo FA, Ayong LS, Agbor GA, Guantai AN. Anti-malarial activity of a polyherbal product (Nefang) during early and established Plasmodium infection in rodent models. Malaria journal. 2014;13(1):456.

97. Jiménez-Díaz MB, Viera S, Ibáñez J, Mulet T, Magán-Marchal N, Garuti H, et al. A new in vivo screening paradigm to accelerate antimalarial drug discovery. PloS one. 2013;8(6):e66967.

98. Chikezie PC, Ibegbulem CO, Mbagwu FN. Bioactive principles from medicinal plants. Research Journal of Phytochemistry. 2015;9:88-115.

99. El-Lamey TM. Effect of Salinity on Tannins Content of Leucaena leucocephala (Lam.) de Wit. and Prosopis chilensis (Molina) Stuntz and Techniques for Their Reduction. Egypt J Bot. 2012:51-63.

100. Ferreira JF, Luthria DL, Sasaki T, Heyerick A. Flavonoids from Artemisia annua L. as antioxidants and their potential synergism with artemisinin against malaria and cancer. Molecules. 2010;15(5):3135-70.

101. O'neill PM, Barton VE, Ward SA. The molecular mechanism of action of artemisinin—the debate continues. Molecules. 2010;15(3):1705-21.

102. Arrey Tarkang P, Nwachiban Atchan AP, Kuiate J-R, Okalebo FA, Guantai AN, Agbor GA. Antioxidant potential of a polyherbal antimalarial as an indicator of its therapeutic value. Advances in pharmacological sciences. 2013.

103. Pandey A, Tripathi S. Concept of standardization, extraction and pre phytochemical screening strategies for herbal drug. Journal of Pharmacognosy and Phytochemistry. 2014;2(5).

104. Akumu EO. PHYTOPHARMACOLOGICAL EVALUATION OF LANTANA CAMARA LEAVES'SMOKE. 2014.

105. Ugochukwu SC, Arukwe Uche I, Ifeanyi O. Available online at www. pelagiaresearchlibrary. com. Asian Journal of Plant Science and Research. 2013;3(3):10-3.

106. Christian AG, Ahunna AG, Nwakaego EM, Chimsorom CK, Chile AE. Antimalarial potential of the ethanolic leaf extract of Pseudocedrala kotschyi. Journal of Acute Disease. 2015;4(1):23-7.

107. Edeoga H, Okwu D, Mbaebie B. Phytochemical constituents of some Nigerian medicinal plants. African journal of biotechnology. 2005;4(7):685-8.

108. Iraqui P, Borah D, Kardong D, Yadav R. Qualitative and Quantitative Screening of Phytochemicals of Meliosomma pinnata (Dermi), A Forest Based Vegetable Plant Traditionally Used by Mising Community of Assam, India. Int J Pharm and Pharma Sc. 2013;5(2):200-3.

Annex

Test for phenols

The plant's solvent extract was put in a test tube and treated with 3-4 drops of 2% of FeCl3. Formation of bluish black/ bluish green coloration indicated the presence of phenols(103, 104).

Test for saponins

Foam Test - 0.5 gm of extract was shaken with 2 ml of water. If foam produced persists for ten minutes it indicates the presence of saponins(103).

Test for alkaloids

1-Wagner's Test: Filtrates were treated with Wagner's reagent (Iodine in Potassium Iodide). Formation of brown/reddish precipitate indicates the presence of alkaloids(103).

2- **Mayer's Test:** a few ml of extract was mixed with 2 drops of Mayer's reagent by adding at the side of the test tube, white creamy precipitate indicates the presence of alkaloids.

Test for Flavonoids (Alkaline reagent test)

2ml of extracts was treated with few drops of 20% sodium hydroxide solution. Formation of intense yellow colour, which becomes colourless on addition of dilute hydrochloric acid, indicates the presence of flavonoids (105).

Test of anthraquinones

50mg of the test compound was diluted in 2 ml of chloroform, homogenized and filtered. Next, 1 ml of 10% NaOH was added to 1 ml of the filtrate. The presence of anthraquinones was indicated by the appearance of a red coloration (106).

Test for Tannins (Braymer's test)

2mls of extract was treated with 10% alcoholic ferric chloride solution and observed for formation of blue or greenish color indicates presence of tannins(105).

Test for terpenoids (Salkowski test):

Five ml of the extract was mixed in 2 ml of chloroform, and 3ml concentrated H2S04 was carefully added to form a layer. A reddish brown colouration of the inter face was formed to show positive results for the presence of terpenoid (107).

Test for cardiac glycosides (Keller-Killani test): Five ml of each extracts was treated with 2 ml of glacial acetic acid containing one drop of ferric chloride solution. This was underlayed with 1 ml of concentrated sulphuric acid. A brown ring of the interface indicates a deoxysugar characteristic of cardenolides. A violet ring may appear below the brown ring, while in the acetic acid layer, a greenish ring may form just gradually throughout thin layer (107).

Test for Steroids-1mL of extracts was dissolved in 10 ml of chloroform and equal volume of concentrated H2SO4 was added by sides of the test tube. The upper layer showed yellow with green fluorescence. This indicates the presence of steroids (108).